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PRINCIPAL INVESTIGATOR: Louis Dubeau, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, California 90089-1147

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Louis Dubeau, M.D., Ph.D.

E-Mail: ldubeau@usc.edu

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14. ABSTRACT
Women with germline mutations in BRCA1 are strongly predisposed to cancers of the ovary and fallopian tubes. Given the strong linkbetween menstrual activity and risk of ovarian cancer in the general population, we hypothesized that BRCA1 might predispose toovarian cancer indirectly, by influencing ovarian granulosa cells, which play an important role in controlling menstrual cycleprogression. We used the Cre-lox system to inactivate the mouse Brca1 gene in granulosa cells. A truncated form of the FSH receptorpromoter was used as Cre driver. Our most recent results show that a majority (40 of 59) of mutant mice develop grossly visible cystictumors either attached to the ovary or the uterine horns. These tumors resembled human serous cystadenomas, which are benign tumorsmade up of the same cell type as ovarian serous carcinomas. We confirmed that these tumors carried only the wild type allele of thefloxed Brca1 allele while the mutant form was present in granulosa cells. These findings strongly support our initial hypothesis thatBrca1 influences tumor development cell non-autonomously, through an effector secreted by granulosa cells. We developed tools suchas long-term cultures of human granulosa cells, which will be used to compare the gene expression patterns of wild type and mutantgranulosa cells in the second year. We also obtained preliminary data suggesting that the dynamics of the hormonal changes associatedwith the estrous cycle are slightly different in mutant mice, suggesting that the influence of granulosa cells on tumor predisposition inthis animal model may be mediated through their role in the ovulatory cycle. Finally, we show evidence that the mutant mice showincreased proliferative activity in epithelial cells lining the uterus and endometrium and endometrial glands, strongly supporting ourview that ovarian epithelial tumors are derived from components of the mullerian tract.

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Introduction

Most individuals with familial predisposition to ovarian carcinoma carry a germline mutation in the *BRCA1* gene. In spite of all the efforts aimed at unerstanding the normal function of the BRCA1 gene product during the last decade, the reason for the association between *BRCA1* mutations and disease predisposition are still unclear. In particular, there is no good explanation for the site specificity of the cancers that develop in these individuals. Indeed, although the *BRCA1* gene is expressed in most cell types, mutation carriers develop primarily breast and ovarian/fallopian tube tumors. We hypothesized that BRCA1 controls ovarian cancer predisposition in a cell non-autonomous manner, through a factor secreted by ovarian granulosa cells. The idea is that reduction in BRCA1 activity in granulosa cells results in changes in the secretion, by those cells, of one or several circulating or paracrine factors that influence the cell of origin of ovarian tumors. We tested this hypothesis by inactivating the *Brca1* gene in mouse ovarian granulosa cells specifically. We had reported, in our initial grant application, that over 50% of the mice carrying this targeted gene knockout developed ovarian/tubal tumors morphologically very similar to human ovarian serous cystadenomas in strong support of our hypothesis. We proposed to elucidate the mechanism of tumor predisposition in this mouse model by identifying the signaling molecules downstream of Brca1 that control tumorigenesis (aim #1) and to test the hypothesis that tumor development in this animal model results from an effect of Brca1 on the epithelial cells lining the entire mullerian tract (aim #2). This latter hypothesis has important implications on the understanding of the exact site of origin of human ovarian epithelial tumors.

Body

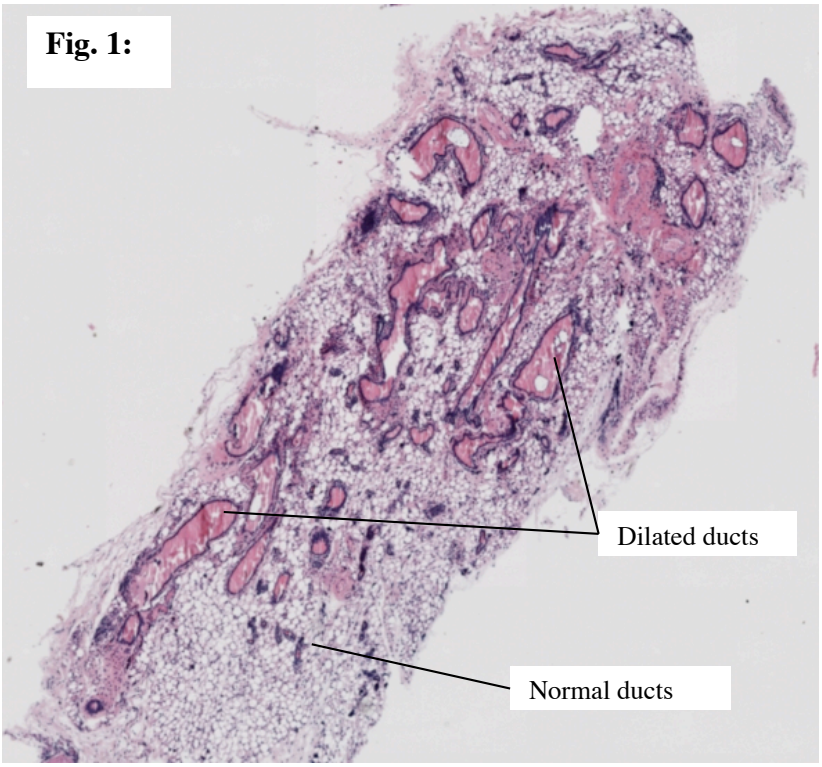
The progress related to each task mentioned in the original statement of work is summarized below:

Task 1: Breed and genotype mice to support aims #1 and #2. According to our initial statement, this task was essentially meant to maintain our mouse colonies in order to secure enough mice to support the other tasks. This was continued during year 2, primarily in support of aim #2. We also continued to examine the phenotype of mutant *versus* wild type mice and, in that regard, expanded the number of wild type (control) mice examined in order to increase the strength of our argument. As of January 2006, 40 of 59 mutant mice developed cysts either in the ovary or uterus while only two of 56 littermate controls developed uterine cysts. The cysts seen in those two control mice were smaller and less numerous than those seen in mutant mice. We therefore maintain our earlier conclusion that targeted inactivation of Brca1 in our mouse model influences proliferation of epithelial cells along the entire mullerian tract. We reported these results and stated that conclusion in a manuscript that was under revision at the time of our last progress report, but has since been published in Current Biology (Curr Biol 15:561-565, 2005). The potential impact of this work is underscored by the fact that it was featured in the News & View section of Nature, April 14, 2005 issue.

Given that humans with germline BRCA1 mutations are predisposed to breast carcinoma in addition to cancers of the mullerian tract, we also started examining the mammary glands of wild type and mutant animals. The number of mammary glands examined is still small, as we first needed to train ourselves at finding and examining this organ, especially since we wanted to examine older mice, where mammary glands are often atrophic and not readily seen. We examined the fourth mammary

gland pair in eight mutant and eight wild type mice with ages ranging between 12 and 18 months. All mice used in this study were virgins. Mammary glands from three of the mutant mice showed dilated breast ducts filled with proteinaceous fluid alternating with areas showing small, inactive ducts. The remaining mutant and all wild type mice showed only small inactive ducts. A representative section of dilated ducts from one mutant is shown in the photomicrograph shown in Fig. 1. The present of such cysts is clearly abnormal and reminiscent of fibrocystic desease in humans. The fact that mutant mice develop abnormalities in the breast in addition to the reproductive tract argues strongly in favor of the relevance of our animal model to humans.

Fig. 1:



Task #2: Test specific candidate hormones for their potential regulation by BRCA1 in vivo. The original plan was to measure circulating levels of various hormones at specific stages of the estrous cycle, which we proposed to evaluate based on the color of the vaginal mucosa. It turned out that this method of determining the stage of the estrous cycle is very inaccurate and subjective. As indicated in our previous progress report, we therefore trained ourselves to determine the stage of the estrus cycle from cytological examination of vaginal lavages stained with Papanicoulou (PAP) stains. We took advantage of this method to expand this task. We reasoned that we should complement our studies aimed at measuring circulating hormone levels with studies aimed at comparing the length of each phase of the estrus cycle in wild type *versus* mutant mice. We had not proposed these studies in our initial application because we did not have the knowledge and technical expertise to do this at that time. We have since examined the length of the various phases of the cycle in a group of wild type and mutant 7-8 month old mice. We also collected serum samples from each mouse. The estrus cycle length was calculated based of daily examinations of vaginal smears over 2-3 weeks. The mice have almost reached the age of 14 months, our next time point. Our plan is to re-examine the mice at that age. The mice will then be sacrificed and their reproductive organs will be examined. We will therefore be able to correlate the presence or absence of ovarian and uterine tumors with estrus cycle abnormalities and circulating hormone levels obtained from 2 different time points. Although this experiment is not completed because we still need to examine and sacrifice the mice at 14 months, the results available so far show a statistically significant elongation of the diestrus and proestrus phases of the cycle in mutant mice as summarized in the following Tables:

Table 1:

	WILD TYPE			
Mouse #	D+P	M	TOTAL	D+P/M
542	2.75	1.75	5.5	1.57
561	2.5	2	6	1.25
574	2.5	1	5	2.5
579	3	1.5	5.5	3
580	2.5	1.8	5	1.39
583	3.25	2.25	6	1.44
584	3.5	3.5	8.5	1
590	3.5	2	6	1.75
Average	2.9375	1.975	5.9375	1.7375
SD	0.43813729	0.72308861	1.11603571	0.67552625

Table 2:

	MUTANT			
Mouse #	D+P	M	TOTAL	D+P/M
490	4.75	1.5	7.5	3.17
491	8	2	11	4
492	9	1.5	11.5	6
430	2	1.5	4.5	1.33
419	4.5	2	7	2.25
432	2.25	1.5	5	1.5
408	6	2	9	3
494	7	2	10	3.5
493	2.5	1.5	4	1.67
Average	5.11111111	1.72222222	7.72222222	2.93555556
SD	2.57424477	0.26352314	2.82965447	1.48373777

The results of Table 1 and 2 revealed that the average combined length of diestrus + proestrus (corresponding to the follicular phase in human) was 2.9 in wild type mice compared to 5.1 in mutant mice ($P = 0.03$, t test). There was no significant difference in the average length of metestrus (corresponding to luteal phase in human) between the two groups. By the end of year 3, we should know whether mice with abnormal cycles are the ones most likely to develop cystic tumors. Although we have collected serum samples from the 7-8 month old mice used for this study, we have not

measured hormone levels yet because we elected to wait until samples from the 14 months time point are collected in order to perform all measurements in parallel.

We repeated the above experiment with 3-4 month old mice, which we plan to re-examine at 7-8 months and 14 months. Again, serum samples will be collected and the mice will be sacrificed at the end of the study in order to determine whether mice with abnormal cycles are most likely to develop cysts in their reproductive tract. The results at 3-4 months are now available and confirm the findings of Table 1 and 2 that mutant mice have a longer cycle due primarily to an increase in the length of diestrus and proestrus. The length of the diestrus phase at 3-4 months was 4.2 +/- 2.0 in mutant mice *versus* 2.3 +/- 1.0 in controls (P = >04) and the length of proestrus was 2.5 +/- 0.9 in mutant *versus* 1.5 +/- 0.6 in controls (P = .02). We anticipate to publish the results in a high profile peer reviewed journal once this study is completed, focussing the discussion on the role of the ovulatory cycle on ovarian tumor predisposition.

Task #3: Test specific candidate hormones for their potential regulation by Brca1 in vitro. The initial proposal was to remove both ovaries from one mutant mouse and one control mouse, harvest granulosa cells, and initiate several granulosa cell cultures per mouse in vitro. After ensuring purity of our cell cultures and verifying their authenticity, we would measure and compare the levels of hormones secreted in the conditioned medium in normal *versus* mutant mice. We have succeeded in obtaining cultures of granulosa cells as initially proposed. We were able to obtain primary cultures of such cells by using sterile needles to puncture ovarian follicles and aspirating the cell rich fluid with a syringe, followed by dispersing the cells and plating them in tissue culture dishes. We showed, in our previous progress report, that the cultured cells expressed mullerian inhibiting substance and developed a strategy to look at the consequences of various hormones of interest on Brca1 expression. Unfortunately, in spite of much effort, we were unable to obtain conclusive results because of a problem that we were not familiar with initially and that we had not anticipated. It turns out that once in culture, granulosa cells, whether from human or mice, differentiate and stop expressing receptors for steroid and gonadotropin hormones. We have contacted reproductive biologists, who were only able to confirm that this is a problem. Our next strategy, which is being pursued during the third year, is to treat mice with high levels of hormones *in vivo* and to look at Brca1 expression by immunohistochemistry and real time PCR in granulosa cells 24 hours post treatment. We realize that this is quite different than our original aim, but those changes in our approach were necessary and should provide us with information on the response of Brca1 to hormones of interest, which was our original intention.

Task s#4 and #5: Expression microarray analyses to compare gene expression in normal and mutant granulosa cells. We needed to modify our approach for the same reason quoted under "Task #3". Our initial strategy relied on the use of cultured granulosa cells isolated from wild type and mutant ovaries as starting material. Once we became familiar with the magnitude of the changes that granulosa cells undergo once put in culture, we realized that our proposed studies would have very little impact if carried out as initially proposed. In response, we now propose to use microdissected cells from frozen mutant and wild type mouse ovaries. This has in part become possible because of novel technologies that were not yet available to us at the time our grant application was first submitted. Affymetrix has developed chips allowing analysis not only of the 3'-end of mRNA species, but of all exons. The human chips (HuEx) have just been released and although it will be another few months before the murine chips (MuEx) are released, these are already available to us via the Early

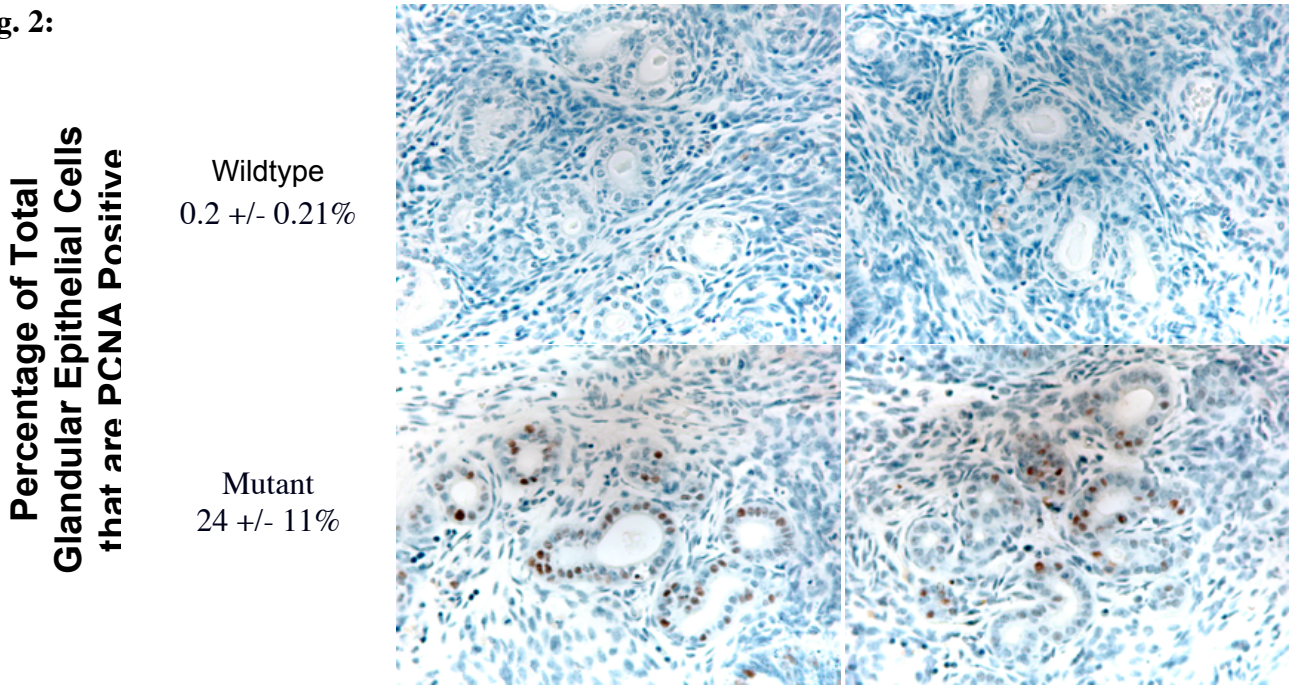
Access Program. There are several advantages to using these chips as opposed to the older U133 series. First, they evaluate an entire gene as opposed to its 3'-end and therefore provide exon level usage and expression data. Second, they are compatible with partly degraded DNA while success with the U133 series was dependent on the availability of intact, high molecular weight RNA. Finally and of great importance to this application, the HhuEx chips have been validated for studies using as little as one ng of RNA as starting material, making this technology readily applicable to cells microdissected from tissue sections. Indeed, if we consider that the average amount of total mRNA per cell to be around 0.1 ng, than using 200 cells per experiment, which is readily obtainable through laser capture microdissection, should provide us with more than sufficient material for our proposed analyses.

Our decision to use this novel technology is not only driven by the fact that we encountered unanticipated problems with our original approach. Even without these problems, we would probably have elected to change our strategy and use the all exon arrays for our proposed experiments in an effort to adapt to technological progress. It is increasingly clear that the 20,000 or so annotated mouse and human genes are in fact static representations of vastly more complex patterns of exon usage in different circumstances. The all exon arrays will allow analysis of both of gene expression (as a virtual gene or "metagene", representing averaged expression values over all consensus exons ascribed to that gene) and exon usage (by evaluation of expression of each exon linked to that gene). It will also be possible to detect aberrant expression of other exons and anonymous genes by data mining of the 1.4 million exons present on these arrays; only about half are accounted for by known gene sequences. Importantly, this includes non-coding RNAs such as micro RNAs and any other expressed RNA sequence found in public databases as of 2004.

Task #6: Comparing proliferation of specific mullerian-derived tissues and ovarian tissues in mutant versus normal mice. Our original plan was to compare the proliferation rate of various tissues of interest in the reproductive tract in normal and mutant mice. We made progress toward this goal during the second year by comparing cellular proliferation activity in the uterus of 5 wild type and 5 mutant mice at the diestrus ad estrus phases of the estrus cycle. Histological cross-sections were taken from each horn at the junction between the proximal (closest to the ovary) and middle thirds. All mice were 3-4 months old. The sections were stained with antibodies against PCNA, a marker of cell cycle activity. A digital photomicrograph of representative areas of each uterus was taken under 40X objective and the total number of endometrial stromal cells showing either positive or negative nuclear staining for PCNA was determined. In accordance with the results shown in last year's progress report, there was a 50% increase in the average percentage of PCNA positive glandular epithelial cells in mutant mice, although there was great variation among the different samples, including one mutant sample that showed almost total lack of PCNA staining, and the results were not statistically significant. There were significant differences in the endometrial stroma. An average of 716 +/- 60 stromal cells were examined in the 5 mutant mice and of 846 +/- 154 cells in the 5 wild type mice. The percentage of PCNA positive stromal cells was 67.8 +/- 6.7% in mutant compared to 36.1 +/- 25% in wild type mice (P = .025, t test). This provides further support for the idea that measurable phenotypic differences exist between cells derived from the mullerian tract in wild type *versus* mutant animals in addition to raising interesting issues about the importance of epithelial-stromal interactions in the uterus. Given that a wild type Brca1 was expressed in the uterus of mutant animals, the results also provide additional support a cell non-autonomous mechanism. We have already set up experiments to examine other stages of the estrus cycle and plan to use other markers listed in the application.

Another set of experiments performed in year 2 investigated differences in cellular proliferation in the uterus of wild type *versus* mutant mice in animals synchronized with regard to their estrus cycles. In the experiment shown in Fig. 2, two mutant mice and two wild type mice were inoculated with PMSG to stimulate follicular growth, followed 48 hours later by inoculation of hCG to induce ovulation. The mice were sacrificed 24 hours after receiving the latter hormone and the uteri were examined for PCNA immunoreactivity. The total number of glandular cells in the entire uterine sections was determined and the percentage of cells that were positive for PCNA was calculated. As shown in the figure, there were practically no glandular cells that were positive for this marker in the wild type mice. In contrast, an average of 24% of the glandular cells in mutant mice were positive. These results need to be regarded as preliminary until a larger number of mice is examined. However, the sharp differences between mutant and wild type animals are striking.

Fig. 2:



Task 7: Comparing proliferation of uterine cysts in mutant mice ovariectomized at specific time points. Although we have initiated these experiments, the results are not yet available because we want to wait until the mice are 14 months old before sacrificing them in order to evaluate their phenotype.

Key Research Accomplishments since last progress report

- We showed that mice carrying a mutant *Brca1* in their granulosa cells have a longer estrus cycle due primarily to increases in he length of the diestrus and proestrus phases. This is interesting in light of extensive epidemiological data showing that continuous (uninterrupted) menstrual cycles in humans are associated with increased ovarian cancer risk.
- We showed preliminary data suggesting that mice carrying a mutant *Brca1* in their granulosa cells show increased cell proliferation in the endometrial stroma as well as in endometrial

glandular epithelium, at least at specific phases of the estrus cycle. A similar increase in endometrial glandular proliferation was seen in mutant mice treated with Pregnant Mare Serum Gonadotropins (PMSG) compared to normal mice ttreated with the same dose of PMSG.

- We revised our strategy for our gene expression profiling studies (Task #4) and our studies on hormonal regulation by Brca1 (Task #3) in light of unanticipated problems with our original approach as well as in an effort to adapt to recent technological progress.

Reportable Outcomes

A manuscript describing our mouse model and arguing that Brca1 controls cancer predisposition in a cell non-autonomous manner was published in Current Biology (Curr Biol 15:561-565, 2005). This manuscript was under review at the time of our last progress report. The potential impact of this work is underscored by the fact that it was featured in the News & View section of Nature, April 14, 2005 issue.

Since our last progress report, Dr. Dubeau was invited to present the work performed in the context of this grant in two symposia, including a symposium on "Ovarian Cancer: Prevention and detection of the Disease and its recurrence" held in Pittsburgh, PA in October 2005 and an International Conference on Ovarian cancer to be held in Crete (Greece) in June 2006. The official handout for the Pittsburgh symposium and the official abstract for the Crete symposium are appended.

Conclusions

Our results confirm the preliminary data presented in our initial grant application and continue to provide strong support, based on a novel experimental mouse model, for the idea that the reason why individuals with germline mutations in the *BRCA1* gene are predisposed to ovarian cancer is that the ensuing decrease in *BRCA1* gene dosage results in a disruption of normal cellular interactions between ovarian granulosa cells and the cells from which ovarian epithelial tumors originate. In other words, BRCA1 controls the secretion of one or several hormonal or paracrine factor(s) by granulosa cells that can influence ovarian tumor predisposition. We further characterized the phenotype of the mutant mice during the second year of this grant by showing that mutant mice have increased endometrial stromal and glandular proliferation at specific stages of their estrus cycle as well as following treatment with gonadotropin hormones. Our most significant progress during the second year was the demonstration that mutant mice have longer estrus cycles, due primarily to an increase in diestrus and proestrus, which correspond to the preovulatory phases of the human menstrual cycle characterized by unopposed estrogen stimulation. This is intriguing in light of the vast epidemiological data showing a strong correlation between menstrual cycle activity and increased risk of ovarian cancer.

References

None.

Appendices

Response to reviewer's comments

Manuscript:

Chodankar R, Kwang S, Sangiorgi F, Hong H, Yen H-Y, Deng C, Pike MC, Shuler CF, Maxson R, Dubeau L: Inactivation of Brca1 in mouse ovarian granulosa cells causes serous epithelial cystadenomas carrying functional Brca1alleles in the ovary and uterus. Curr Biol 15:561-565, 2005.

Text of a News & Views article that appeared in the April 14, 2005 issue of Nature.

Handout of lecture given at Symposium entitled: "Ovarian Cancer: Prevention and Detection of the Disease and its Recurrence" held in Pittsburgh, PA on October 24-25, 2005

Abstract of a lecture given at the "1st International Conference on Ovarian Cancer: State of the Art and Future Directions" held in Crete (Greece) from June 26 to July 1, 2006

Revised Statement of Work

Response to reviewer's comments

The original progress report was modified in response to reviewer's comments and comply to specific requests. To facilitate review of this revised report, this section indicates, point by point, how each of the reviewer's concerns were addressed.

The PI listed invited seminar and symposia presentations of the work resulting from this project as Reportable Outcome but failed to append abstracts (with full details) as required by USAMRMC

There were no official abstracts for the Pittsburgh meeting. However, an official handout containing the slides used during the presentation was given to all participants. I have appended this handout to the revised progress report. There was an official abstract that was part of the meeting material given to all participants at the Crete meeting. This abstract has also been appended. In retrospect, I realize that the seminar given at University of Virginia should not have been listed as reportable outcome because it was simply a lecture given in the context of a seminar series at this institution. There were no written documents released. I have deleted this seminar from the section on "Reportable Outcomes" in the revised report.

This reviewer recommends, at a minimum, a modification of the Statement of Work (SOW) and revised scope of the work

A revised statement of work, which reflects the changes mentioned in the progress report under tasks 3-4, has been appended to the revised report. Task 5, which is a verification of the microarray data, will be performed based on data obtained from dissected granulosa cells as opposed to cultured granulosa cells. However, this task remains essentially the same as the original task 5.

In Task 4 the PI plans to microdissect frozen embryos and will use HuEX and MuEX chips for microarray analysis. This reviewer is uncertain if the proposed experiments will be done on human or mouse frozen embryos. The PI has not been granted approval to use human substances.

The only reason why the HuEx chip was mentioned in the original progress report was because this is the only exon array chip that was commercially available at the time of submission of the report and we wanted to make the point that this technology was available. Our plan all along has been to use the MuEx chip on mutant versus wild type mice. This chip has now been released and is available to us. We have modified the body of the progress report in the paragraph describing tasks #4 and #5 in order to clarify that our proposed studies will be done on mice.

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Cell-Nonautonomous Induction of Ovarian and Uterine Serous Cystadenomas in Mice Lacking a Functional Brca1 in Ovarian Granulosa Cells

Rajas Chodankar,[1] Stanford Kwang,[4] Frank Sangiorgi,[2] Hao Hong,[1] Hai-Yun Yen,[2] Chuxia Deng,[5] Malcolm C. Pike,[3] Charles F. Shuler,[4] Robert Maxson,[2] and Louis Dubeau[1,*]

[1]Department of Pathology
[2]Department of Biochemistry
[3]Department of Preventive Medicine
University of Southern California/Norris Comprehensive Cancer Center
[4]Center for Craniofacial Molecular Biology
University of Southern California
Los Angeles, California 90033
[5]Genetics of Development and Diseases Branch
National Institute of Diabetes and Digestive and Kidney Diseases
Bethesda, Maryland 20892

Summary

Women with germline mutations in *BRCA1* have a 40% risk of developing ovarian cancer by age 70 [1] and are also predisposed to cancers of the fallopian tubes [2–4]. Given that ovulatory activity is a strong risk factor for sporadic ovarian cancer [5], we hypothesized that reduced BRCA1 expression might predispose to gynecological cancers indirectly, by influencing ovarian granulosa cells. These cells secrete sex steroids that control the ovulatory cycle and influence the growth of ovarian epithelial tumors [6, 7]. Granulosa cells also secrete mullerian inhibiting substance (MIS), a hormone that inhibits both the formation of female reproductive organs in male embryos [8] and the proliferation of ovarian epithelial tumor cells [9, 10]. We tested this hypothesis by using the *Cre-lox* system to inactivate the *Brca1* gene in mouse ovarian granulosa cells. A truncated form of the Fsh receptor promoter [11] served as the Cre driver. Here, we show that indeed, inactivation of the *Brca1* gene in granulosa cells led to the development of cystic tumors in the ovaries and uterine horns. These tumors carried normal *Brca1* alleles, supporting the view that Brca1 may influence tumor development indirectly, possibly through an effector secreted by granulosa cells.

Results

Granulosa Cell Specificity of Truncated Fsh Receptor Promoter

We verified the cell-type specificity of a truncated Fsh receptor promoter form shown previously to direct expression exclusively in granulosa cells [11]. We crossed a transgenic mouse expressing the Cre recombinase under the control of this promoter fragment with the ROSA26R Cre reporter mouse strain [12]. Examination of the pelvic organs of mice carrying the Cre driver and reporter showed β-galactosidase activity exclusively in granulosa cells (Figure 1).

Consequences of Loss of Brca1 in Granulosa Cells on Tumor Formation

Fshr-Cre transgenic mice were crossed with mice carrying a floxed *Brca1* allele [13] to create a *Brca1* homozygous knockout restricted to granulosa cells. One ovary was removed from each of 30 *Brca1 flox/flox; Fshr-Cre* mice at 2 months of age. Histological examination revealed that these ovaries were morphologically normal (not shown). The mice were fertile and, at least during the first 6 months of life, produced litters of normal size.

Fifty-nine *Brca1 flox/flox;Fshr-Cre* mice, including the 30 mice that had a unilateral oophorectomy at two months, were sacrificed between the ages of 12 and 20 months. Of these 59, 40 (68%) homozygous mutant mice had grossly visible cysts attached to the ovary, within the wall of the uterine horns, or on the external surface of the uterine horns (Figure 2). The ovarian cysts were occasionally bilateral (Figure 2). The uterine cysts were usually multiple and most concentrated near the ovaries. All cysts were lined by cuboidal to columnar cells and were occasionally papillary (see Figure 3E, below). The cysts resembled human serous cystadenomas, which are benign tumors composed of the same cell type as ovarian serous carcinomas.

A solid tumor contiguous to a morphologically benign cyst was observed in a single case. Although the complexity and cellular atypia levels seen in the solid component were compatible with a malignant process, the malignant potential of this tumor remains unclear because it showed no evidence of either invasion of surrounding structures or metastasis (Figure 2D). Renal cysts were also observed in two mutant mice. No abnormality was seen in any of 36 age-matched littermate controls lacking *Cre* recombinase.

Evidence for a Cell-Nonautonomous Mechanism of Tumor Induction

Our studies with the R26R reporter mouse (Figure 1) suggested that rearrangement of the *Brca1* gene in response to Fshr-Cre occurred primarily in ovarian granulosa cells, our intended target. That all tumors exhibited an epithelial morphology suggested that they were not derived from granulosa cells. Further support for this possibility came from findings that the tumor cells (1) expressed keratins (Figure 3), which are markers of epithelial cells, and (2) did not express mullerian inhibiting substance, a marker of granulosa cells (Figure 3). The tumor cells also expressed estrogen (Figure 3) and progesterone (not shown) receptor proteins, further supporting the view that they were functionally similar to human ovarian epithelial tumors.

The conclusion that the tumors did not originate in granulosa cells was also supported by the fact that they were often localized in the uterine horns, which do not contain granulosa cells. The possibility remained

*Correspondence: ldubeau@usc.edu

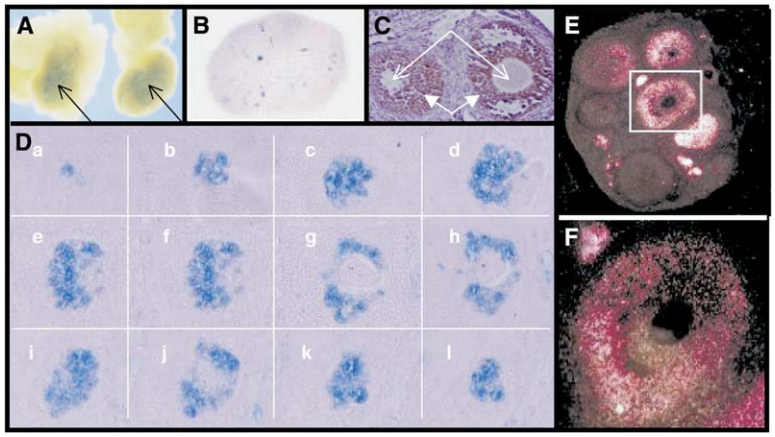

Figure 1. Specificity of Fsh Receptor Promoter for Granulosa Cells

Two transgenic mouse lines expressing Cre recombinase under the control of a truncated form [11] of the Fsh receptor promoter (285 bp) were crossed with a ROSA26R Cre reporter mouse. The pelvic organs were removed at 8 weeks postnatal and examined for the presence of *lacZ* under bright-field (A, B, and D) or dark-field (E and F) microscopy. Shown here are representative results from one transgenic line.

(A) Ovaries with portion of adjacent uterine horns; *lac*Z staining is restricted to the ovaries (arrows).

(B) Whole-mount section of one ovary showing scattered foci of *lac*Z.

(C) Histological section of a mouse ovary stained with an antibody against mullerian inhibiting substance, a marker of granulosa cells; this panel is meant to illustrate the normal histology of ovarian follicles for use as reference when examining panels (D)–(F); it shows two ovarian follicles (short arrows), each with a central oocyte (long arrows) surrounded by immunopositive granulosa cells. Cells outside the two ovarian follicles are ovarian stromal cells.

(D) Serial sections through an entire ovarian follicle morphologically similar to those shown in (C) and showing *lac*Z staining confined to the granulosa cells.

(E) Whole-mount section through an entire ovary seen under dark-field microscopy showing the presence of *lac*Z in ovarian follicles; the area within the rectangle, which shows a cross-section through the center of one follicle as well as small portions of adjacent follicles, is enlarged in (F).

that the Fshr-Cre transgene produced a Cre level that was sufficient to cause recombination of the floxed *Brca1* allele but was too low to cause recombination of the R26R allele. If this were the case, then the tumor cells should carry the recombined form of the floxed *Brca1* allele. However, although the expected 530 bp product from the unrearranged *Brca1* allele could be amplified readily from all tissues examined with PCR primers specific for this allele, the only tissues from which the expected 640 bp product from the rearranged allele could be amplified were whole ovaries, the site of granulosa cells, as well as one of four ovarian cysts that had been separated from the adjacent ovaries with scissors under a dissecting microscope (Figure 4, bottom panel). The weak amplification product obtained with primers specific for the rearranged allele (pair e-d) in this ovarian cyst most likely reflects the presence of admixed ovarian stroma, either in the cyst wall or in contaminating fragments of normal ovary. It is highly unlikely that this allele played a role in tumor development owing to its absence in most cystic tumors examined. A fifth ovarian cyst, subjected to laser capture microdissection to ensure absence of admixed granulosa cells, did not contain the rearranged allele either in the lining epithelium or in the cyst wall (Figure 4, middle panel). We detected only the unrearraged allele in the epithelial lining of two additional uterine cysts examined after laser capture microdissection (not shown).

Discussion

Our results strongly support our hypothesis that inactivation of the *Brca1* gene in granulosa cells acts cell-nonautonomously by altering the activity of an effector that influences tumorigenesis in cells from which ovarian epithelial tumors originate. This conclusion is based on the fact that inactivation of *Brca1* in ovarian granulosa cells led to the formation of epithelial tumors carrying normal *Brca1* alleles. An earlier report showed similarly that breast tumors resulting from inactivation of *Brca1* in a subset of mammary cells (with MMTV-Cre or Wap-Cre) did not carry the mutant form of *Brca1* [13]. Although we did not examine breast tissue in *Brca1 flox/flox;Fshr-cre* mice, we note that ovulatory activity, which is controlled largely by the activity of granulosa cells, is a well-established risk factor for breast cancer in humans [14].

That mice lacking a functional Brca1 protein in their granulosa cells developed lesions involving the uterine horns in addition to the ovaries is consistent with the observation that precancerous changes are frequently seen in the fallopian tubes of women who are asymptomatic carriers of *BRCA1* mutations [2–4]. This is also compatible with L.D.'s earlier suggestion that ovarian epithelial tumors do not originate from the mesothelial layer lining the ovarian surface, the site favored by most authors, but from mullerian duct derivatives surrounding the ovary or abutting this organ [15].

Most tumors that develop in individuals with germline *BRCA1* mutations and show loss of heterozygosity at the *BRCA1* locus have retained the mutant allele [16–18], suggesting that BRCA1 may act as a classical tumor suppressor. However, not all tumors that develop in this group of patients carry losses of heterozygosity at this locus [18], and there is little evidence for the notion that the wild-type allele in these tumors is silenced by epigenetic mechanisms [19]. In addition, a number of observations suggest that total loss of BRCA1 activity does not promote, but interferes with cellular growth. The small number of breast or ovarian cancer cell lines so far isolated that lack a functional BRCA1 protein typically have long doubling times. Primary cultures derived from *Brca1*$^{-/-}$ mouse embryos

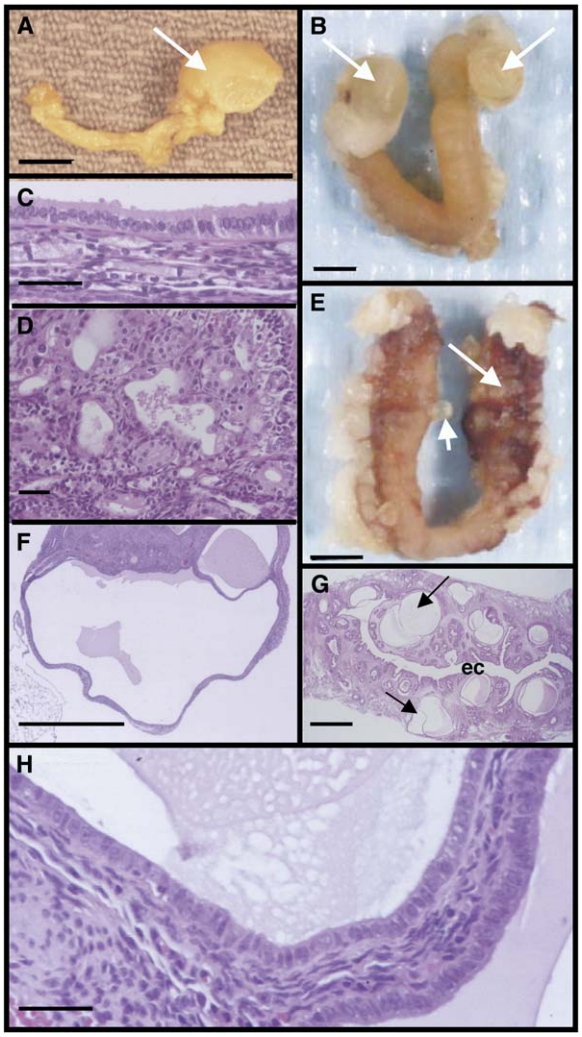

Figure 2. Examples of Ovarian and Uterine Lesions Observed in Mutant Mice

Shown are gross photographs of ovarian (arrows in [A] and [B]) and uterine (arrows in [E]) cysts and histological sections from ovarian (C and D) and uterine (F–H) lesions. The ovarian tumor shown in (A) was 80% cystic and 20% solid. Histological sections of both of these components are shown in (C) and (D), respectively. A bilocular cyst on the external surface of a uterine horn is shown under low and high magnification in panels (F) and (H), respectively. A uterine horn containing multiple epithelial cysts (arrows) is shown at low magnification in (G); ec denotes endometrial cavity. The scale bars in (A), (B), and (E) represent 5 mm. The scale bars in (C), (D), and (H) represent 40 μm. The scale bars in (F) and (G) represent 1000 μm. Stain: hematoxylin and eosin.

do not proliferate unless the embryos also carry a p53 knockout. Given that cells from such embryos grow only clonally, additional events must occur to ensure their viability [20, 21]. Recent evidence suggests that downregulation of BRCA1 results in growth arrest at the G2 to M transition [22], a finding clearly inconsistent with the view that Brca1 functions as a classical tumor suppressor. That mutations in this ubiquitously expressed gene lead mainly to predisposition to breast and ovarian cancer is also difficult to reconcile with this view.

It is possible that the Fshr promoter used in our studies is expressed in cells other than granulosa cells at levels undetectable with the R26R reporter mouse. Thus, non-granulosa cells may control ovarian and uterine tumorigenesis. However, we favor the hypothesis that it is the granulosa cells that act at a distance to control mullerian epithelial tumorigenesis via a mechanism regulated by Brca1. At least in reproductive organs, these cells appear to be the principal site of Brca1 inactivation. The idea that a specific effector released by granulosa cells and regulated by Brca1 influences tumor predisposition in the mullerian tract is both the simplest and biologically most attractive hypothesis that follows from our data. Another possibility is that an abnormal Brca1 expression might result in alterations in the dynamics of the estrus cycle. An example would be changes in the length of a specific phase of this cycle. Such changes, in turn, might influence tumor predisposition.

The finding that loss of Brca1 in mouse ovarian granulosa cells causes epithelial tumors in wild-type cells of the ovary and uterus raises the prospect that reduced levels of functional BRCA1 protein in humans carrying a germline *BRCA1* mutation could lead to the development of cancer by modulating the ability of granulosa cells to act on distant target tissues. This hypothesis has important implications for the clinical management of individuals with a familial predisposition to ovarian tumors owing to germline *BRCA1* mutations. Knowing the identity of the endocrine or paracrine factor(s) that mediates such actions at a distance could provide a new way to identify individuals predisposed to ovarian cancer and could also form the basis for novel strategies based on manipulations of the levels of the factor(s) in question and aimed at preventing ovarian cancer in individuals with familial predisposition to this disease.

Experimental Procedures

Immunohistochemical Analyses

The mouse monoclonal antibody against nonsquamous keratins was purchased from Chemicon International (Temecula, CA, MAB 1611). The polyclonal goat antibody against mouse mullerian inhibiting substance was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, catalog number sc-6886). Goat polyclonal antibodies against mouse estrogen receptor α and progesterone receptor proteins were purchased from Santa Cruz Biotechnology (catalog numbers sc-542 and sc-2018, respectively). All primary antibodies were diluted 1:200. For secondary antibodies, we used either anti-mouse IgG purchased from Chemicon International (catalog number AP124B) diluted 1:500 or anti-goat IgG purchased from Santa Cruz Biotechnology and diluted 1:200. Antibody binding was detected with the ABC kit (Vector Laboratories, Burlingame, CA).

Examination of *Brca1* Rearrangement Status by PCR

Tissues of interest were either microdissected with a Pixcell II laser-capture microdissection instrument (Arcturus Bioscience, Mountain View, CA) or were sampled under a dissecting microscope. All laser-capture microdissections were performed on tissues fixed in ethanol only, embedded in paraffin, and either unstained or lightly stained with hematoxylin. Genomic DNA was amplified by PCR with primers specific for either the unrearranged (primers a-b) or rearranged (primers e-d) alleles. The sequences of primers a and b were as published [13]. The sequence of primer e (forward) was: 5′-GCAGTGAAGAGAACTTGTTCCT-3′. The se-

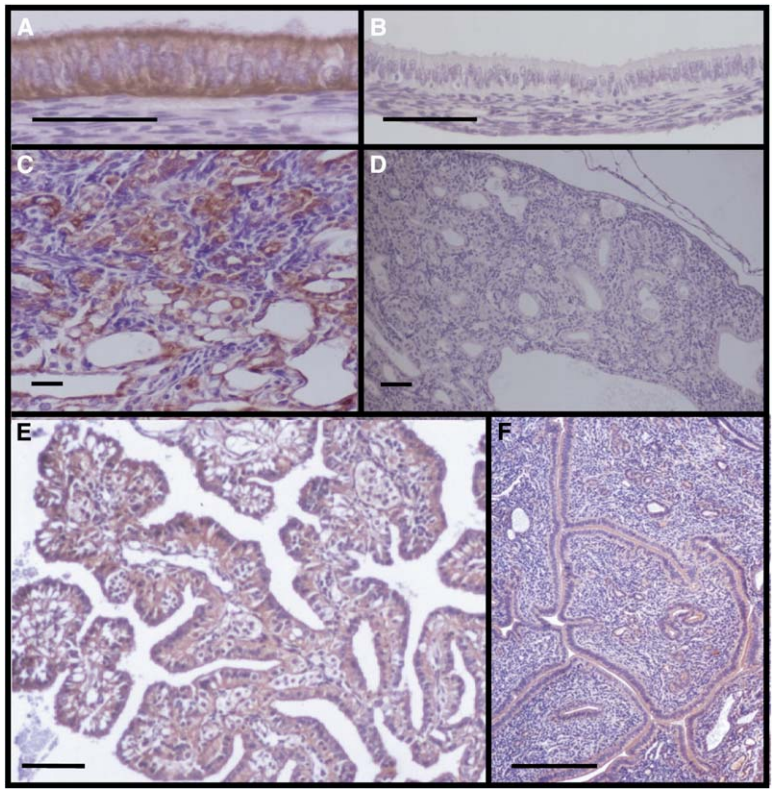

Figure 3. Immunohistochemical Characterization of Ovarian Tumors

(A)–(E) are various portions of the ovarian tumor shown in Figure 2A. (E) shows a papillary area of the cystic component. The sections were stained with a polyclonal antibody against nonsquamous keratins (A and C) and with monoclonal antibodies against either mullerian inhibiting substance (B and D) or the estrogen receptor protein (E). The cytoplasmic staining pattern seen in (E) is similar to that seen in sections of normal endometrium from wild-type mice (F). Secondary mouse ovarian follicles stained with an antibody against mullerian inhibiting substance were shown in Figure 1C. The scale bars represent 40 μm in all panels except (E), in which it is 100 μm.

quence of primer d (reverse) was: 5′-CTGCGAGCAGTCTTCAG AAAG-3′. PCR cycling profiles were 30 s at 94°C, 60 s at 58°C, and 60 s at 72°C over 35 cycles.

Generation of Mice with *Brca1* Knockout Targeted to Granulosa Cells

A transgene composed of the Cre recombinase protein-coding sequence (1.1 kb) and a 900 bp SV40 fragment containing an untranslated exon sequence and polyadenylation signal fused with a truncated form [11] of the FSH receptor promoter (285 bp) was used to produce transgenic mice. The initial parental mice were from a cross between C57 and Black 6 strains. Six lines were initially created, two of which were crossed with R26R reporter mice and found to be equally effective at driving *Cre*. One line was selected randomly for breeding with a mouse carrying a floxed *Brca1* allele described earlier [21]. The mouse genotypes were determined by amplifying tail DNA with primers specific for either the floxed *Brca1* allele or for *Cre*.

Acknowledgments

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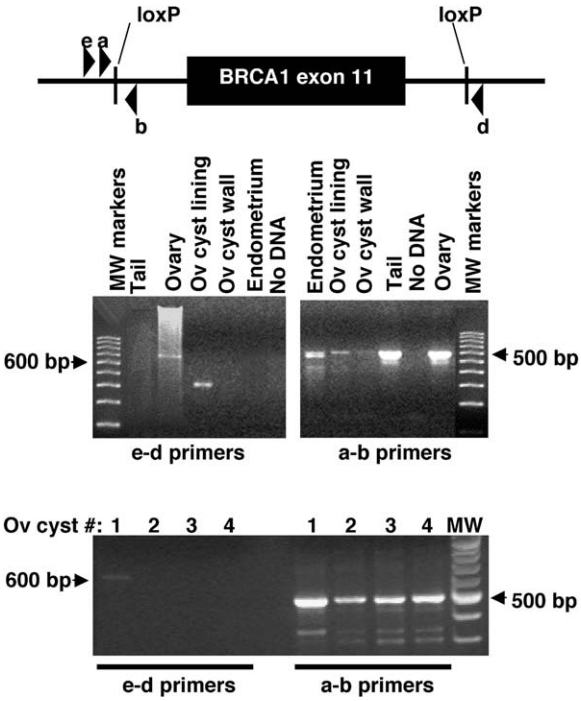

Figure 4. Recombination Status of Floxed Brca1 Alleles in Normal and Neoplastic Tissues

The top diagram shows *lox*P sites flanking exon 11 of the *Brca1* gene. The arrowheads represent the position and orientation of oligonucleotide primers in relation to the *lox*P sites. Genomic DNA from various normal or neoplastic tissues was amplified by PCR with either primer pair e-d, specific for the rearranged *Brca1* allele, or pair a-b, specific for the unrearranged allele. The PCR products were resolved on agarose gels and visualized under UV after staining with ethidium bromide (middle and bottom panels). Lanes labeled "Ov cyst lining" and "endometrium" in the middle panel represent, respectively, the epithelial lining of an ovarian cyst and of endometrium separated from adjacent tissues by laser-capture microdissection. The stromal cells underlying the ovarian cyst epithelium were likewise microdissected and examined ("Ov cyst wall"). The bottom panel shows four ovarian cysts not subjected to laser-capture microdissection but separated from the adjacent ovaries with scissors under a dissecting microscope. Although the expected 530 bp unrearranged *Brca1* allele (primer pair a-b) was detected in all tissues examined, the expected 647 bp amplification product from the rearranged allele (primer pair e-d) was only seen in normal ovarian tissues (which contain granulosa cells) as well as in one of the four ovarian cysts not subjected to microdissection (bottom panel). The product of approximately 300 bp in "Ov cyst lining" (primer pair e-d, middle panel) was sequenced, confirming that it represents a nonspecific product. The 647 bp product from normal ovaries with the same primers was also sequenced and shown to be authentic.

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Cancer

Remote control

Curr. Biol. **15,** 561–565 (2005)

BRCA1 is notorious as the first gene to be linked with inherited susceptibility to breast and ovarian cancer. It has been thought of as a classic 'tumour suppressor', but Rajas Chodankar *et al.* suggest that it may have another, more subtle, effect.

Granulosa cells in the ovary produce the sex hormones that regulate the ovulatory cycle — and the growth of ovarian tumours. Given that repeated ovulations (that is, fewer pregnancies or reduced oral contraceptive use) are known to increase the risk of non-hereditary ovarian cancer, the researchers wondered whether decreased levels of BRCA1 protein in granulosa cells are involved. Using mice, they inactivated the gene specifically in these cells. The animals developed tumours in the ovaries and uterine horns. But the tumour cells looked like epithelial cells and had normal copies of the gene, implying that they had not developed from granulosa cells.

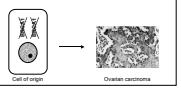
Inactivating *BRCA1* seems, therefore, to be controlling some intermediary produced by the granulosa cells. It is this unidentified factor that appears to promote tumours in the ovary epithelium, so providing a lead for further investigation. **Helen Dell**

MECHANISM OF OVARIAN CANCER PREDISPOSITION IN INDIVIDUALS WITH GERMLINE *BRCA1* MUTATIONS

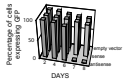
Louis Dubeau, M. D., Ph. D.
USC/Norris Comprehensive Cancer Center
Keck school of Medicine of University of Southern California

MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS

Tumor suppressor hypothesis:

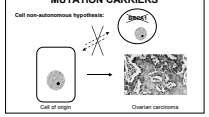


SELECTIVE DISADVANTAGE OF REDUCED *BRCA1* EXPRESSION



MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS

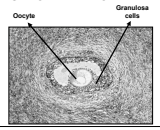
Cell non-autonomous hypothesis:



INFLUENCE OF PARITY AND ORAL CONTRACEPTIVE USE ON OVARIAN CANCER RISK

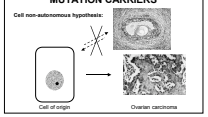
Risk Factors		Relative Risk
Parity	0	1.00
	1	0.81
	2	0.42
	3	0.55
	4+	0.31
Oral Cont.	0	1.0
Contraceptive	<1	0.9
Use (yr)	1-3	0.8
	3-5	0.5
	5+	0.4

SECONDARY FOLLICLE

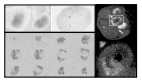


MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS

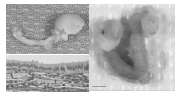
Cell non-autonomous hypothesis:



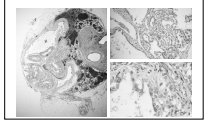
DISTRIBUTION OF Fshr PROMOTER ACTIVITY IN R26R MICE



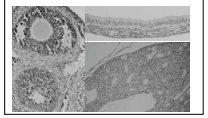
MUTANT MICE DEVELOP OVARIAN CYSTADENOMAS



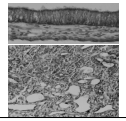
INVASIVE CARCINOMA IN A P53/BRCA1 DOUBLE MUTANT



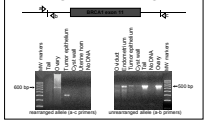
IMMUNOHISTOCHEMICAL STAINING FOR MULLERIAN INHIBITING SUBSTANCE



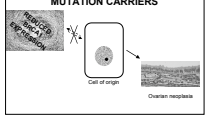
IMMUNOHISTOCHEMICAL STAINING FOR NON-SQUAMOUS KERATINS



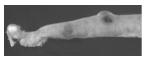
TISSUE DISTRIBUTION OF MUTANT ALLELES



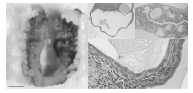
MECHANISM OF OVARIAN CANCER PREDISPOSITION IN BRCA1 MUTATION CARRIERS



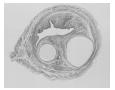
PERI-TUBAL CYST IN BRCA1 KNOCK-OUT MICE

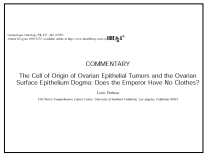


MUTANT MICE DEVELOP EPITHELIAL CYSTS ALONG THE ENTIRE MULLERIAN TRACT



UTERINE CYST





COMMENTARY

The Cell of Origin of Ovarian Epithelial Tumors and the Ovarian Surface Epithelium Dogma: Does the Emperor Have No Clothes?

Louis Dubeau
USC/Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033

Embryological Development of the Female Reproductive System (9 weeks)

http://www.med.unc.edu/embryo_images/unh-welcome/welcome_htms/contents.htm

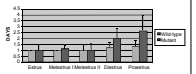
7

POSSIBLE MECHANISMS FOR OVARIAN TUMOR PREDISPOSITION BASED ON A CELL NON-AUTONOMOUS SCENARIO

- Direct mechanism
  - An effector secreted by granulosa cells is regulated by Brca1
- Indirect mechanism
  - Brca1 inactivation results in alterations in the ovulatory cycle, which in turn influences ovarian epithelial tumor development

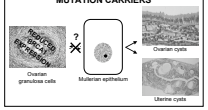
8

COMPARISON OF LENGTH OF ESTRUS CYCLE STAGES IN MUTANT *VERSUS* WILD-TYPE LITTERMATE MICE



9

CELL NON-AUTONOMOUS MECHANISM OF PREDISPOSITION TO CANCERS OF THE REPRODUCTIVE TRACT IN BRCA1 MUTATION CARRIERS



Modulation of Aromatase Expression by BRCA1: a Possible Link to Tissue-Specific Tumor Suppression

Yanfen Ha[1], Sagar Ghosh, Asma Ardeshi, Wei Yao,[1] Yuanzhe Lu,[1] Adam Katz,[2] Rong Li[1,3]

[1]Department of Biochemistry and Molecular Genetics
[2]Department of Medicine and Division of Endocrinology
[2]Department of Plastic and Reconstructive Surgery
School of Medicine
University of Virginia
Charlottesville, VA 22908

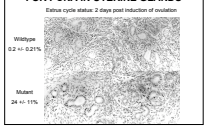
[3]State Key Laboratory of Genetic Engineering
Institute of Genetics
School of Life Science
Fudan University
Shanghai 200433, China

Oncogene, in press

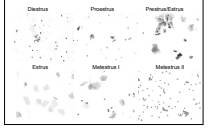
SUMMARY

- The embryonic lethality of BRCA1 general knock out was avoided by knocking out this gene in granulosa cells specifically
- Mouse carrying mutant alleles of BRCA1 in their granulosa cells had morphologically normal ovarian follicles and were fertile, but developed benign and malignant epithelial ovarian tumors as well as extra-ovarian cysts in their mullerian tract
- Alterations in interactions between granulosa cells and the cell of origin of ovarian epithelial tumors may be the key to familial ovarian cancer predisposition in individuals carrying germline BRCA1 mutations.

IMMUNOHISTOCHEMICAL STAINING FOR PCNA IN UTERINE GLANDS



Classification of estrus cycle stages in vaginal smears using the Papanicolaou stain



SIGNIFICANCE

- A better understanding of the normal interactions between ovarian granulosa cells and the cell of origin of ovarian epithelial tumors may lead to novel strategies for the identification of individuals at risk and for ovarian cancer prevention.
- The results support the hypothesis that ovarian epithelial tumors are of mullerian origin, which is important for the understanding of their precursor lesions and for the development of effective screening strategies for their early detection.

MAMMARY GLAND OF 1.5 YEAR OLD VIRGIN MUTANT MOUSE

ACKNOWLEDGEMENTS

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- Axel Schönthal (USC)
- French Anderson (USC)
- Nori Kasahara (USC/UCLA)
- Chu-Xia Deng (NIH)

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Aegean Conferences Abstract Submission Form

IMPORTANT

1) Please ensure that you have filled out the Title, Authors, Affiliations and Abstract sections.
2) The maximum number of words allowed in your entire abstracts 400 words. This includes the Title, Author(s), Affiliation(s) AND Abstract body.
3) Save the completed for upload using Microsoft Word 2000 or lower version.

I. Contact Information:

Name:	Louis Dubeau
Address:	USC/Norris Comprehensive Cancer Center #7320 1441 Eastlake Avenue
City:	Los Angeles
State:	CA
Zip Code:	90033
Country:	United States of America
Telephone:	(323) 865-0720
Fax:	(323) 865-0077
E-mail:	ldubeau@usc.edu
Meeting:	1st International Conference on Ovarian Cancer: State of the Art and Future Directions

II. Abstracts

IMPORTANT: Please select the grey text below *(by clicking once on the grey text)* and start typing in the designated section. The text is pre-formatted in Times New Roman 12pt. font single line spacing. You may alter these values only to add symbols or superscripts etc. Use Symbol font for Greek and other special characters.
Do **NOT** include tables, figures or references. These will be removed before your abstract is published.

Title:
BRCA1 INDUCED OVARIAN ONCOGENESIS

Louis Dubeau

University of Southern California Keck school of Medicine
USC/Norris Comprehensive Cancer Center

Women with germline mutations in *BRCA1* have a 40% risk of developing ovarian cancer by age 70 and are also predisposed to cancers of the fallopian tubes and breast. Although some observations are supportive of the currently favored notion that BRCA1 functions as a classical tumor suppressor gene, others are inconsistent with this notion. Given that ovulatory activity is a strong risk factor for sporadic ovarian cancer, we hypothesized that reduced BRCA1 expression might predispose to gynecological cancers indirectly by influencing ovarian granulosa cells. These cells secrete sex steroids that control the ovulatory cycle and influence the growth of ovarian epithelial tumors. We tested this hypothesis by using the *Cre-lox* system to inactivate the *Brca1* gene in mouse ovarian granulosa cells. A

truncated form of the FSH receptor promoter served as the Cre driver in order to achieve such tissue specificity. Indeed, inactivation of the *Brca1* gene in granulosa cells led to the development of benign cystic tumors in the ovaries and uterine horns in approximately one third of the mutant mice. The mutant mice also developed epithelial cysts in their mammary glands. These tumors were of epithelial origin and carried normal *Brca1* alleles, supporting the view that Brca1 influenced their development indirectly, through an effector secreted by granulosa cells. The fact that the cystic tumors were not confined to the ovaries, but involved the entire mullerian tract, is also consistent with the view that ovarian tumors are not of mesothelial origin, but are derived from the mullerian tract. We measured the length of each phase of the estrus cycle in a group of mutant and wild type mice at 3-4 months and 7-8 months of age. In both age groups, there was a significant increase in the length of proestrus, which corresponds to the follicular phase of the human menstrual cycle. We suggest that the increased cancer predisposition seen in individuals carrying a germline *BRCA1* mutation is due, at least in part, to reduced levels of BRCA1 expression in granulosa cells. This, in turn, may lead to alterations in the length of the follicular phase of the menstrual cycle and ensuing increased unopposed estrogen stimulation as well as, perhaps, to disruption of additional interactions between granulosa cells and mullerian epithelial cells resulting in promotion of neoplastic transformation.

Task #1: Breed and genotype mice to support aims #1 and #2. Breed FSHR/cre mice with floxed BRCA1 mice and perform genotyping analyses of offspring. Mice with homozygous rearrangements of the BRCA1 gene will be used as "mutant" mice. Mice with no rearrangement will be used as "control" mice. Mice with heterozygous rearrangements will be discarded. A total of 222 mice will be needed to support aims #1 and of 258 to support aim #2.
Period: months 1-36.
Outcome: enough control and mutant mice will be generated to support our proposed studies.

Task #2: Test specific candidate hormones for their potential regulation by BRCA1 in vivo (aim #1). Draw 200 microliters of blood from 25 mutant mice and from 25 control mice10-12 months of age on the second day of the estrus cycle (determined from examination of color changes on the vaginal mucosa). Collect serum and send to Dr. Stanczyk's lab for measurement of levels of estradiol, estrone, and progesterone. Keep 25 microliters of each serum sample for measuring MIS by ELISA in Dr.Dubeau's laboratory. Sacrifice mice after blood is collected and examine ovaries for MIS levels by immunohistochemistry. (months 1-3, repeat during months 4-7).
Period: months 1-3, repeat during months 4-7.
Outcome: the consequences of BRCA1 mutation on specific hormones of interest in mice will be determined.

Task #3: Test specific candidate hormones for their potential regulation by BRCA1 in vivo. Inoculate 3-4 month old wild type mice obtained from same litters as our Brca1 knock out mice with PMSG followed, 48 hours later, by inoculation of either hCG or vehicle control. Sacrifice the mice and examine the ovaries for expression of Brca1 by immunohistochemistry and real-time RT-PCR. Five mice will be used in each group. This study will be repeated a second time to ensure reproducibility.
Period: months 30-36.
Outcome: Knowledge of the effects of reproductive hormones on Brca1 expression, used in conjunction with the observation that loss of Brca1 activity in granulosa cells predisposes to ovarian tumor development, will shed light on possible explanations for the well-established link between the menstrual cycle and ovarian cancer predisposition.

Task #4: Expression microarray analyses to compare gene expression in normal and mutant granulosa cells. Examine vaginal cytology in mutant and littermate controls at 3-4 months and 7-8 months in order to identify animals at the proestrus phase of their cycle and sacrifice 5 mice in each group at this phase of the cycle. Obtain frozen sections of the ovaries and use laser capture microdissection to microdissect about 500 granulosa cells from secondary ovarian follicles. Extract total RNA from each ovary and pool RNA samples from each group. Give samples to Genomics Core Facility for preparation of cDNA, hybridization to MuEx expression microarray chips, and data analysis. Repeat once.
Period: months 30-36.
Outcome: Comprehensive knowledge of the signaling pathways affected by inactivation of BRCA1 in granulosa cells will be acquired. This will provide independent verification

of the conclusions from tasks #2 and #3 and will increase our understanding of the consequences of BRCA1 mutations.

Task #5: Verification of microarray data. Gene products of interest thought to be upregulated or downregulated in mutant mice based on microarray data will be analyzed either by quantitative RT-PCR (using a TaqMan 7700 instrument available in Dr. Dubeau's lab) or by immunohistochemistry and western blotting (depending on availability of suitable antibodies) in cultured normal versus mutant granulosa cells from 10-12 week old mice or in sections of ovarian tissue. If necessary due to lack of suitable alternative methods, in situ hybridization will be used. We anticipate that up to gene products will be tested during the course of he grant.
Period: months 33-36.
Outcome: predictions from task #4 will be verified.

Task #6: Comparing proliferation of specific mullerian-derived tissues and ovarian tissues in mutant versus normal mice (aim #2). Ovaries from 5 normal mice and from 5 mutant mice will be analyzed for histone-3 phosphorylation at 1, 2, 3, 6, and 12 months of age. Number of positive cells in endometrium at the proximal, mid, and distal ends of the uterine horns, rete ovarii, ovarian follicles (pre- and post-estrus), ovarian stroma, and ovarian surface epithelium will be quantitated and compared in both groups.
Period: year 1, repeat in years 2 and 3.
Outcome: The spectrum of tissues affected by BRCA1 mutations will be known, providing insights into the nature of the cell of origin of ovarian epithelial tumors and increasing our understanding of the mechanisms responsible for ovarian cancer predisposition in individuals carrying BRCA1 mutations.

Task #7: Comparing proliferation of uterine cysts in mutant mice ovariectomized at specific time points (aim #2). Mice will be ovariectomized at 1, 2, 6, and 12 months of age (5 mice will be obtained for each age group) and the number of cysts on the external uterine surface as well as cell proliferation (evaluated based on histone-3 phosphorylation) in the epithelial cells lining the cysts will be measured at 12 months of age.
Period: year 1, repeat in years 2 and 3.
Outcome: we will know whether the effects of BRCA1 alterations are cumulative over long time periods or are most important at specific time points such as when mice reach sexual maturity; we will also learn about the reversibility of the changes associated with BRCA1 mutations.

Task #8: Collection of serum samples from normal women and BRCA1 carriers (aim #3). Serum samples from thirty 25-39 year old BRCA1 carriers and 30 matched controls will be collected by Dr. John Hopper and shipped to us.
Period: years 1-2.
Outcome: material needed for experiments proposed in aim #3 will be available.

Task #9: Measurement of MIS and sex steroid hormones in serum of normal and BRCA1 carrier women (aim #3). Measurement of estradiol, estrone, and progesterone levels will

be done in the laboratory of Dr. Stanczyk. Measurements of MIS levels will be done by ELISA in Dr.Dubeau's laboratory.
Period: year 3.
Outcome: our hypothesis that women with germline BRCA1 mutations have altered levels of either MIS or of MIS:sex steroid hormone ratios will be tested.